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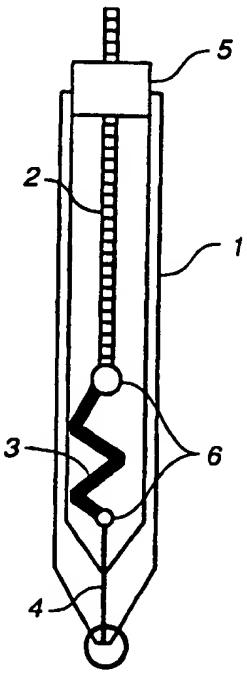
(54) Title: DETECTION AND DIFFERENTIATION OF BACTERIA USING ELECTRICAL DETECTION METHODS



(57) Abstract: The present invention provides an apparatus and methods for the electrical detection of bacterial cells. Specifically, the present invention provides an apparatus and methods for the electrical detection of viable bacteria. The present invention further provides an apparatus and methods for differentiating between multiple bacterial cell types using electrical detection techniques.

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DETECTION AND DIFFERENTIATION OF BACTERIA USING ELECTRICAL DETECTION METHODS

FIELD OF THE INVENTION

This invention relates to an apparatus and methods for electrical detection of bacterial cells. Specifically, the invention relates to an apparatus and methods for the electrical detection of viable bacteria. The invention further relates to an apparatus and methods for differentiating between multiple bacterial cell types using electrical detection techniques.

BACKGROUND OF THE INVENTION

Bacterial detection techniques conventionally have been based on standard immunological or molecular methods that rely upon optical, fluorescent, radioactive, or enzyme-linked detection. There are, however, disadvantages in the use of such techniques, which adversely affect cost, simplicity, and reproducibility. Standard immunological bacterial detection methods, for example, require at least one labeled antibody and often a significant investment in expensive equipment for detecting interactions between bacterial cells in a sample mixture and fluorescently- or radioactively-labeled antibodies. Furthermore, immunological methods do not permit differentiation of viable bacteria from bacterial cell debris, an important requirement in assays designed to detect the presence of trace amounts of a viable, health-threatening bacterial cells in a sample mixture. Molecular bacterial detection methods, such as the polymerase chain reaction, also are costly and technically complex, because of the need for specific equipment and because optimization of reaction conditions specific for a particular bacterial strain can be laborious and time-consuming. Such techniques also suffer from the disadvantage of being unable to easily detect viable bacteria from bacterial cell debris.

Bacterial detection methods utilizing electrical or electrochemical detection strategies present one possible alternative to methods that rely on standard immunological and molecular detection strategies. Electrical and electrochemical detection techniques are based on the detection of changes in electrical parameters such as current, potential, or dielectricity arising from electrochemical reactions, interactions between one group of molecules attached to the surface of an electrode (often referred to as "probe" molecules) and another set of molecules present in a testing sample (often referred to as "target" molecules) or other physical or chemical changes in the testing system. Electrical detection eliminates many of

the disadvantages inherent in using radioactive or fluorescent labels to detect interactions between the probe and target molecules. Electrical detection is safe, inexpensive, and sensitive, and is not burdened with complex and onerous regulatory requirements.

Methods for electrochemical detection of viable bacteria using immobilized anti-bacterial antibodies have been previously disclosed. Perez *et al.*, 1998, *Anal. Chem.* 70:2380-86, disclose an electrochemical bacterial detection technique using antibodies tethered to magnetic beads that capture bacteria from a matrix, followed by reaction of the antibody-bacterial complexes with redox mediators (potassium hexacyanoferrate(III) and 2,6-dichlorophenolindophenol). Viable bacteria are detected by the transfer of electrons from the cellular respiratory chain to redox mediators. Electrochemical measurement of the reduced mediator is then carried out by flow injection analysis and amperometric methods.

While the bacterial detection method of Perez *et al.* permits differentiation of viable bacteria from bacterial cell debris, the technique has the disadvantage of requiring that electrochemical mediators be used. As with standard immunological techniques requiring labeled antibodies, this results in experimental assays having an increased cost and complexity.

Thus, there remains a need in the art to develop inexpensive, reliable and safe alternatives to current bacterial detection methods. Suitable alternatives to currently available techniques will be capable of differentiating between viable bacteria and bacterial cell debris. A suitable alternative to currently available techniques will also permit bacterial detection of viable bacteria without requiring electrochemical reporters. The development of such methods has wide application in such areas as disease diagnostics, environmental studies, food safety analyses, and pathogen detection.

SUMMARY OF THE INVENTION

The present invention provides an apparatus and methods for the electrical detection of bacterial cells. The apparatus and methods of the invention are useful for detecting bacteria and differentiating between different bacterial strains using specific binding molecules and electrical detection techniques.

The apparatus and methods of the present invention are useful for detecting viable bacteria in a sample mixture by electrical detection of interactions between specific binding

molecules, such as antibodies, bound to defined regions of an ordered array and viable bacterial target molecules in the sample mixture that come in contact with the specific binding molecules. The apparatus and methods of the present invention are further useful for differentiating bacterial cells of a particular bacterial species, subspecies, or strain in a sample mixture. As provided herein, different bacterial species, subspecies or stains are differentiated by electrical detection of interactions between the particular bacterial cells in the sample mixture and specific binding molecules that have specificity for the bacterial cells of the particular bacterial species, subspecies, or strain and that are bound to defined regions of an ordered array.

The apparatus of the present invention comprises a supporting substrate, one or a plurality of microelectrodes in contact with the supporting substrate, one or a plurality of linking moieties in contact with the microelectrodes and to which specific binding molecules are immobilized, at least one counter-electrode in electrochemical contact with the microelectrodes, a means for producing an electrical signal at each microelectrode, a means for detecting changes in the electrical signal at each microelectrode, and an electrolyte solution in contact with the microelectrodes, linking moieties, and counter-electrode.

The apparatus of the present invention may advantageously further comprise at least one reference electrode. The apparatus may also further comprise a plurality of wells, each of which encompasses at least one microelectrode in contact with a linker moiety and at least one counter-electrode that is sufficient to interrogate the microelectrodes in contact with linker moieties. The apparatus may still further comprise a means for killing the bacteria in the sample mixture.

The apparatus and methods of the present invention are useful for the electrical detection of bacteria in a sample mixture. In one method of the present invention, an electrical signal is detected in a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized. Using the method, the plurality of microelectrodes in contact with linker moieties bearing immobilized specific binding molecules is then exposed to a sample mixture containing bacterial cells, and an electrical signal is detected at the plurality of the microelectrodes. The electrical signal detected before and after exposing the plurality of microelectrodes to the sample mixture is compared, and bacterial cells in the sample mixture are detected by determining the difference in the

electrical signal measured before and after exposing the microelectrodes to the sample mixture.

The apparatus and methods of the present invention are also useful for the electrical detection of viable bacteria in a sample mixture. In another method of the present invention, a plurality of microelectrodes in contact with linker moieties bearing immobilized specific binding molecules is exposed to a sample mixture containing viable and non-viable bacterial cells. A first electrical signal is detected at the plurality of microelectrodes, and the bacteria in the sample mixture are then killed. A second electrical signal is then detected at the plurality of microelectrodes. The electrical signal detected before and after killing the bacteria in the sample mixture is compared, and viable bacterial cells in the sample mixture are detected by determining the difference in the electrical signal measured before and after killing the bacteria in the sample mixture.

The apparatus and methods of the present invention are further useful for the electrical detection of bacterial cells of a particular bacterial species, subspecies, or strain in a sample mixture. In still another method of the present invention, an electrical signal is detected in a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized. The plurality of microelectrodes is then exposed to a sample mixture containing bacterial cells of a particular species, subspecies, or strain, and an electrical signal is detected at the plurality of microelectrodes. The electrical signal detected before and after exposing the plurality of microelectrodes to the sample mixture is compared, and bacterial cells of a particular species, subspecies, or strain in the sample mixture are detected by determining the difference in the electrical signal before and after exposing the plurality of microelectrodes to the sample mixture.

It is an advantage of the present invention that the apparatus and methods provided herein permit electrical detection and differentiation of bacteria without requiring that electrochemical reporters or labeled target molecules be used. As a result, when compared with previously disclosed methods, the present invention provides bacterial detection assays capable of differentiating viable bacteria from bacterial cell debris at both reduced cost and experimental complexity, and having increased reproducibility and sensitivity.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B illustrate a schematic representation of the structure of a microelectrode in contact with a polyacrylamide gel linker moiety (i.e., a porous hydrogel microelectrode) (Figure 1A) and a schematic representation of the structure of the tip of the porous hydrogel microelectrode (Figure 1B);

Figure 2 illustrates a porous hydrogel microelectrode;

Figure 3 illustrates a schematic representation of the interaction between antibodies immobilized on a porous hydrogel microelectrode and a bacterial cell;

Figures 4A-4B are graphical representations of the dependence of measured capacitance with frequency (Figure 4A) and measured resistance with frequency (Figure 4B) for a porous hydrogel microelectrode to which is immobilized a polyclonal anti-*E. coli* antibody before (curve 1) and after (curve 2) incubation with an *E. coli* containing sample;

Figures 5A-5B are graphical representations of the dependence of measured capacitance with frequency (Figure 5A) and measured resistance with frequency (Figure 5B) for a porous hydrogel microelectrode to which is immobilized a polyclonal anti-*E. coli* antibody after incubation with an *E. coli* sample (curve 1), after a subsequent incubation at 65°C (curve 2), or after a subsequent incubation with a HRP-labeled polyclonal anti-*E. coli* antibody (curve 3);

Figures 6A-6B are graphical representations of the dependence of measured capacitance with frequency (Figure 6A) and measured resistance with frequency (Figure 6B) for a porous hydrogel microelectrode to which is immobilized a polyclonal anti-*E. coli* antibody after incubation with an *E. coli* sample (curve 1) or after a subsequent incubation with a HRP-labeled polyclonal anti-*E. coli* antibody (curve 2); and

Figure 7 is a graphical representation of the dependence of measured impedance with frequency for a porous hydrogel microelectrode before (curve 1) and after (curve 2) immobilization of a polyclonal anti-*E. coli* antibody, and following (curve 3) incubation with *B. subtilis* containing sample.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In preferred embodiments of the present invention, the apparatus and methods are useful for detecting viable bacteria in a sample mixture by electrical detection of interactions

between specific binding molecules, such as antibodies, bound to defined regions of an ordered array and viable bacterial target molecules in the sample mixture, which are permitted to come in contact with the specific binding molecules. In other preferred embodiments of the present invention, the apparatus and methods are useful for differentiating bacterial cells of a particular bacterial strain in a sample mixture by electrical detection of interactions between specific binding molecules - having -- specificity for the bacterial cells of the particular bacterial strain -- bound to defined regions of an ordered array and the particular bacterial cells in the sample mixture that come in contact with the specific binding molecules.

As used herein, the term "array" refers to an ordered spatial arrangement, particularly an arrangement of immobilized biomolecules such as specific binding molecules according to this invention. The present system finds particular utility in array formats, i.e. wherein there is a matrix of addressable locations (herein generally referred to "pads", "addresses" or "micro-locations"). By "array" herein is meant a plurality of capture binding probes in an array format; the size of the array will depend on the composition and end use of the array. Arrays containing from about 2 different capture probes to many thousands can be made. Generally, the array will comprise from two to as many as 100,000 or more per cm^2, depending on the size of the pads, as well as the end use of the array. Preferred ranges are from about 2 to about 10,000, with from about 5 to about 1000 being preferred, and from about 10 to about 100 being particularly preferred. In some embodiments, the compositions of the invention may not be in array format; that is, for some embodiments, compositions comprising a single capture binding probe may be made as well. In addition, in some arrays, multiple substrates may be used, either of different or identical compositions. Thus for example, large arrays may comprise a plurality of smaller substrates.

As used herein, the term "addressable array" refers to an array wherein the individual elements have precisely defined x and y coordinates, so that a given element at a particular position in the array can be identified.

As used herein, the terms "probe" and "biomolecular probe" "binding ligand" or "binding molecule" refer to a molecule (preferably a biomolecule) used to detect another biomolecule. Examples include antigens that detect antibodies, oligonucleotides that detect

complimentary oligonucleotides, and ligands that detect receptors. Such probes are preferably immobilized on a microelectrode comprising a substrate.

By "binding ligand" or grammatical equivalents herein is meant a compound that is used to probe for the presence of the target analyte, and that will bind to the analyte.

As will be appreciated by those in the art, the composition of the binding ligand will depend on the composition of the target analyte. Binding ligands for a wide variety of analytes are known or can be readily found using known techniques. For example, when the analyte is a protein, the binding ligands include proteins (particularly including antibodies or fragments thereof (FAbs, etc.)) or small molecules. When the analyte is a metal ion, the binding ligand generally comprises traditional metal ion ligands or chelators Preferred binding ligand proteins include peptides. For example, when the analyte is an enzyme, suitable binding ligands include substrates and inhibitors. Antigen-antibody pairs, receptor-ligands, and carbohydrates and their binding partners are also suitable analyte-binding ligand pairs. The binding ligand may be nucleic acid, when nucleic acid binding proteins are the targets; alternatively, as is generally described in U.S. Patents 5,270,163, 5,475,096, 5,567,588, 5,595,877, 5,637,459, 5,683,867,5,705,337, and related patents, hereby incorporated by reference, nucleic acid "aptomers" can be developed for binding to virtually any target analyte. Similarly, there is a wide body of literature relating to the development of binding partners based on combinatorial chemistry methods. In this embodiment, when the binding ligand is a nucleic acid, preferred compositions and techniques are outlined in PCT US97/20014, hereby incorporated by reference.

By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989),

O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of RAMs or conductive oligomers, or to increase the stability and half-life of such molecules in physiological environments. As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention.

In a preferred embodiment, the binding of the target analyte to the binding ligand is specific, and the binding ligand is part of a binding pair. By "specifically bind" herein is meant that the ligand binds the analyte, with specificity sufficient to differentiate between the analyte and other components or contaminants of the test sample. However, as will be appreciated by those in the art, it will be possible to detect analytes using binding which is not highly specific; for example, the systems may use different binding ligands, for example an array of different ligands, and detection of any particular analyte is via its "signature" of binding to a panel of binding ligands, similar to the manner in which "electronic noses" work.

This finds particular utility in the detection of chemical analytes. The binding should be sufficient to remain bound under the conditions of the assay, including wash steps to remove non-specific binding. In some embodiments, for example in the detection of certain biomolecules, the disassociation constants of the analyte to the binding ligand will be less than about 10^{-4}-10^{-6} M^{-1}, with less than about 10^{-5} to 10^{-9} M^{-1} being preferred and less than about 10^{-7} -10^{-9} M^{-1} being particularly preferred.

In a preferred embodiment binding ligands may be proteins. By "proteins" or grammatical equivalents herein is meant proteins, oligopeptides and peptides, derivatives and analogs, including proteins containing non-naturally occurring amino acids and amino acid analogs, and peptidomimetic structures. The side chains may be in either the (R) or the (S) configuration. In a preferred embodiment, the amino acids are in the (S) or L-configuration. As discussed below, when the protein is used as a binding ligand, it may be desirable to utilize protein analogs to retard degradation by sample contaminants.

In one preferred embodiment Apop antibodies are used as binding ligands. An Apop protein may be used to generate polyclonal and monoclonal antibodies to Apop proteins, which are useful as described herein. The terms "Apop antibodies", "antibodies binding to Apop" or grammatical equivalents thereof include antibodies binding to Apop1 proteins, Apop2 proteins, and Apop3 proteins.

Apop antibodies usually are generated with an Apop protein having the amino acid sequence depicted in Figures 2, 4, and 6. In a preferred embodiment, Apop proteins corresponding to a portion or fragment of an Apop protein of which the amino acid sequence is depicted in Figures 2, 4, and 6, are used to generate antibodies. Methods for the preparation and purification of monoclonal and polyclonal antibodies are known in the art and e.g., are described in Harlow and Lane, *Antibodies: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1988). When the Apop protein is used to generate antibodies, the Apop protein must share at least one epitope or determinant with the full length protein shown in Figures 2, 4, and 6. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to a smaller Apop3 protein will be able to bind to the full length protein.

In a preferred embodiment, the epitope is unique; that is, antibodies generated to a unique epitope show little or no cross-reactivity to other proteins. The term "antibody"

includes antibody fragments, as are known in the art, including Fab Fab_2, single chain antibodies (Fv for example), chimeric antibodies, etc., either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA technologies. The term "antibody" further comprises polyclonal antibodies and monoclonal antibodies, which can be agonist or antagonist antibodies.

The Apop antibodies of the invention specifically bind to Apop proteins. By "specifically bind" herein is meant that the antibodies bind to the protein with a binding constant in the range of at least 10^{-4}- 10^{-6} M^{-1}, with a preferred range being 10^{-7} - 10^{-9} M^{-1}.

In a preferred embodiment the targets are cells. As used herein cells include any number of viruses (including orthomyxoviruses, (e.g. influenza virus), paramyxoviruses (e.g respiratory syncytial virus, mumps virus, measles virus), adenoviruses, rhinoviruses, coronaviruses, reoviruses, togaviruses (e.g. rubella virus), parvoviruses, poxviruses (e.g. variola virus, vaccinia virus), enteroviruses (e.g. poliovirus, coxsackievirus), hepatitis viruses (including A, B and C), herpesviruses (e.g. Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus), rotaviruses, Norwalk viruses, hantavirus, arenavirus, rhabdovirus (e.g. rabies virus), retroviruses (including HIV, HTLV-I and -II), papovaviruses (e.g. papillomavirus), polyomaviruses, and picornaviruses, and the like), and bacteria (including a wide variety of pathogenic and non-pathogenic prokaryotes of interest including Bacillus; Vibrio, e.g. *V. cholerae;* Escherichia, e.g. Enterotoxigenic *E. coli*, Shigella, e.g. *S. dysenteriae;* Salmonella, e.g. *S. typhi;* Mycobacterium e.g. *M. tuberculosis, M. leprae*; Clostridium, e.g. *C. botulinum, C. tetani, C. difficile, C.perfringens*; Cornyebacterium, e.g. *C. diphtheriae*; Streptococcus, *S. pyogenes, S. pneumoniae;* Staphylococcus, *e.g. S. aureus*; Haemophilus, e.g. *H. influenzae*; Neisseria, e.g. *N. meningitidis, N. gonorrhoeae*; Yersinia, e.g. *G. lambliaY. pestis*, Pseudomonas, e.g. *P. aeruginosa, P. putida*; Chlamydia, e.g. *C. trachomatis*; Bordetella, e.g. *B. pertussis;* Treponema, e.g. *T. palladium;* and the like).

As used herein, the terms "bioarray," "biochip" and "biochip array" refer to an ordered spatial arrangement of immobilized biomolecules on a microelectrode arrayed on a solid supporting substrate. Preferred probe molecules include nucleic acids, oligonucleotides, peptides, ligands, antibodies and antigens; peptides and proteins are the most preferred probe species. Biochips, as used in the art, encompass substrates containing arrays or microarrays, preferably ordered arrays and most preferably ordered, addressable

arrays, of biological molecules that comprise one member of a biological binding pair. Typically, such arrays are oligonucleotide arrays comprising a nucleotide sequence that is complementary to at least one sequence that may be or is expected to be present in a biological sample. Alternatively, and preferably, proteins, peptides or other small molecules can be arrayed in such biochips for performing, *inter alia*, immunological analyses (wherein the arrayed molecules are antigens) or assaying biological receptors (wherein the arrayed molecules are ligands, agonists or antagonists of said receptors). In particularly preferred embodiments, the probes of the invention are specific for a particular species, subspecies or strain of bacteria, and most preferably are specific for viable bacteria of said species, subspecies or strain.

In preferred embodiments, the apparatus of the present invention comprises a supporting substrate, one or a plurality of microelectrodes in contact with the supporting substrate, one or a plurality of linking moieties in contact with the microelectrodes and to which specific binding molecules are immobilized, at least one counter-electrode in electrochemical contact with the microelectrodes, a means for producing an electrical signal at each microelectrode, a means for detecting changes in the electrical signal at each microelectrode, and an electrolyte solution in contact with the microelectrodes, linking moieties, and counter-electrode.

In some embodiments of the present invention, the specific binding molecules of the apparatus comprise proteins or peptides. In one preferred embodiment, the specific binding molecules are antibodies. The antibodies immobilized on the linker moieties of the apparatus of the invention may be polyclonal or monoclonal antibodies, F(ab) fragments, F(ab)' fragments, $F(ab)_2$ fragments, or F_v fragments of polyclonal or monoclonal antibodies, or F(ab) or single chain antibodies selected from *in vitro* libraries. In alternative embodiments of the present invention, the specific binding molecules are nucleic acids, oligonucleotides, or combinations thereof. In one preferred embodiment of the present invention, the specific binding molecules are aptamers (*i.e.*, oligonucleotides capable of interacting with target molecules such as peptides). Natural products libraries (such as, *inter alia*, yeast extracts), phage display libraries, or combinatorial libraries may also be used as specific binding molecules.

In one preferred embodiment of the present invention, the specific binding molecules are polyclonal antibodies and the antibodies are immobilized on the linker moieties using any technique known in the art that does not interfere with or inhibit the ability of the antibodies to specifically bind to a bacterial cell expressing or containing its conjugate antigen. In preferred embodiments, polyclonal antibodies, antisera, monoclonal antibodies, fragments of said polyclonal or monoclonal antibodies, or other specific ligand binding molecules, are immobilized onto and attached to the linker moieties of the apparatus of the invention using biotinylated species thereof that are conjugated with streptavidin added to the linker moiety material. In more preferred embodiments, the linker moiety material to which the streptavidin is added is polyacrylamide gel. In other embodiments the biotinylated specific binding molecule is a protein, peptide, nucleic acid, or oligonucleotide.

In some embodiments of the present invention, the linker moieties of the apparatus are composed of materials including, but not limited to, polyacrylamide gel, agarose gel, polyethylene glycol, cellulose gel, or sol gels. In preferred embodiments, the linker moieties comprise polyacrylamide gel, which form a gel pad. In alternative embodiments of the present invention, the linker moieties, or gel pad comprise a conjugated polymer or copolymer film. Such conjugated polymer or copolymer film is composed of materials including, but not limited to, polypyrrole, polythiphene, polyaniline, polyfuran, polypyridine, polycarbazole, polyphenylene, poly(phenylenvinylene), polyfluorene, or polyindole, or their derivatives, their copolymers, and combinations thereof. In preferred embodiments, the linker moieties comprise a neutral pyrrole matrix.

Polymeric hydrogels and gel pads are used as binding layers to adhere biological molecules to substrate surfaces, which biomolecules include, but are not limited to, proteins, peptides, oligonucleotides, polynucleotides, and larger nucleic acid fragments. The oligonucleotide probes may be bound to the surface of a continuous layer of the hydrogel, or to an array of gel pads. The gel pads comprising biochips for use with the apparatus of the present invention are conveniently produced as thin sheets or slabs, typically by depositing a solution of acrylamide monomer, a crosslinker such methylene bisacrylamide, and a catalyst such as N, N, N', N' - tetramethylethylendiamine (TEMED) and an initiator such as ammonium persulfate for chemical polymerization, or 2,2-dimethoxy-2-phenyl-acetophone (DMPAP) for photopolymerization, in between two glass surfaces (e.g., glass plates or

microscope slides) using a spacer to obtain the desired thickness of the polymeric gel. Generally, the acrylamide monomer and crosslinker are prepared in one solution of about 4-5% acrylamide (having an acrylamide/ bisacrylamide ratio of 19/1) in water/glycerol, with a nominal amount of initiator added. The solution is polymerized and crosslinked either by ultraviolet (UV) radiation (e.g., 254 nm for at least about 15 minutes, or other appropriate UV conditions, collectively termed "photopolymerization"), or by thermal initiation at elevated temperature (e.g., typically at about 40° C). Following polymerization and crosslinking, the top glass slide is removed from the surface to uncover the gel. The pore size (and hence the "sieving properties") of the gel is controlled by varying the amount of crosslinker and the percent solids in the monomer solution. The pore size also can be controlled by varying the polymerization temperature.

In the fabrication of arrays of polyacrylamide (i.e., patterned gels), in an embodiment of the present invention, the acrylamide solution typically is imaged through a mask during the UV polymerization/crosslinking step. The top glass slide is removed after polymerization, and the unpolymerized monomer is washed away (developed) with water, leaving a fine feature pattern of polyacrylamide hydrogel, which is used to produce the crosslinked polyacrylamide hydrogel pads. Further, in an application of lithographic techniques known in the semiconductor industry, light can be applied to discrete locations on the surface of a polyacrylamide hydrogel to activate these specified regions for the attachment of an oligonucleotide, an antibody, an antigen, a hormone, hormone receptor, a ligand or a polysaccharide on the surface (e.g., a polyacrylamide hydrogel surface) of a solid substrate (*see, e.g,* WO 91/07087, incorporated herein by reference).

For hydrogel-based arrays using polyacrylamide, biomolecules (such as oligonucleotides) are covalently attached by forming an amide, ester or disulfide bond between the biomolecule and a derivatized polymer comprising the cognate chemical group. Covalent attachment of the biomolecule to the polymer is usually performed after polymerization and chemical cross-linking of the polymer is completed.

Alternatively, oligonucleotides bearing 5'-terminal acrylamide modifications can be used that efficiently copolymerize with acrylamide monomers to form DNA-containing polyacrylamide copolymers (Rehman *et al.*, 1999, *Nucleic Acids Research* 27: 649-655). Using this approach, stable probe-containing layers can be fabricated on substrates (*e.g.*,

microtiter plates and silanized glass) having exposed acrylic groups. This approach has made available the commercially marketed "Acrydite™" capture probes (available from Mosaic Technologies, Boston, MA). The Acrydite moiety is a phosporamidite that contains an ethylene group capable of free-radical copolymerization with acrylamide, and which can be used in standard DNA synthesizers to introduce copolymerizable groups at the 5' terminus of any oligonucleotide probe.

The solid substrate can be made of a wide variety of materials and can be configured in a large number of ways, as is discussed herein and will be apparent to one of skill in the art. In addition, a single device may be comprises of more than one substrate; for example, there may be a "sample treatment" cassette that interfaces with a separate "detection" cassette; a raw sample is added to the sample treatment cassette and is manipulated to prepare the sample for detection, which is removed from the sample treatment cassette and added to the detection cassette. There may be an additional functional cassette into which the device fits; for example, a heating element which is placed in contact with the sample cassette to effect reactions such as PCR. In some cases, a portion of the substrate may be removable; for example, the sample cassette may have a detachable detection cassette, such that the entire sample cassette is not contacted with the detection apparatus. See for example U.S. Patent No. 5,603,351, PCT US96/17116, and "MULTILAYERED MICROFLUIDIC DEVICES FOR ANALYTE REACTIONS" filed in the PCT December 11, 2000, Serial No. PCT/US00/33499, hereby incorporated by reference.

The composition of the solid substrate will depend on a variety of factors, including the techniques used to create the device, the use of the device, the composition of the sample, the analyte to be detected, the size of the wells and microchannels, the presence or absence of electronic components, etc. Generally, the devices of the invention should be easily sterilizable as well.

The supporting substrate of the apparatus of the invention is advantageously made from any solid material, including but not limited to silicon such as silicon wafers, silicon dioxide, silicon nitride, glass and fused silica, gallium arsenide, indium phosphide, aluminum, ceramics, polyimide, quartz, plastics, resins and polymers including polymethylmethacrylate, acrylics, polyethylene, polyethylene terepthalate, polycarbonate, polystyrene and other styrene copolymers, polypropylene, polytetrafluoroethylene,

superalloys, zircaloy, steel, gold, silver, copper, tungsten, molybdeumn, tantalum, KOVAR, KEVLAR, KAPTON, MYLAR, brass, sapphire, etc. High melting borosilicate or fused silicas may be preferred for their UV transmission properties when any of the sample manipulation steps require light based technologies. In addition, as outlined herein, portions of the internal surfaces of the device may be coated with a variety of coatings as needed, to reduce non-specific binding, to allow the attachment of binding ligands, for biocompatibility, for flow resistance, etc. In preferred embodiments, the supporting substrate of the apparatus of the present invention is composed of silicon or glass. The linker moieties are embedded within or placed in contact with the supporting substrate.

In another preferred embodiment, the substrate is made from printed circuit board (PCP) materials, including without limitation, fiberglass, teflon, ceramics, glass, silicon, mica, plastic (including acrylics, polystyrene and copolymers of styrene and other materials, polypropylene, polyethylene, polybutylene, polycarbonate, polyurethanes, Teflon™, and derivatives thereof, etc.), GETEK (a blend of polypropylene oxide and fiberglass), etc.

The supporting substrate has a surface area of from about 0.01μm^2 to about 5 cm^2 containing between 1 and 1 x 10^8 microelectrodes in contact with said linker moieties. In a preferred embodiment, the supporting substrate has a surface area of 10,000 μm^2 and contains 10^4 microelectrodes in contact with linker moieties. In preferred embodiments, the microelectrodes are arranged on the supporting substrate so that they are separated by a distance of from about 0.05μm to 0.5mm. In more preferred embodiments, the microelectrodes are regularly spaced on the solid supporting substrate with a uniform spacing there between.

In general, circuit board materials are those that comprise an insulating substrate that is coated with a conducting layer and processed using lithography techniques, particularly photolithography techniques, to form the patterns of electrodes and interconnects (sometimes referred to in the art as interconnections or leads). The insulating substrate is generally, but not always, a polymer. As is known in the art, one or a plurality of layers may be used, to make either "two dimensional" (e.g. all electrodes and interconnections in a plane) or "three dimensional" (wherein the electrodes are on one surface and the interconnects may go through the board to the other side) boards. Three dimensional systems frequently rely on the use of drilling or etching, followed by electroplating with a metal such as copper, such that

the "through board" interconnections are made. Circuit board materials are often provided with a foil already attached to the substrate, such as a copper foil, with additional copper added as needed (for example for interconnections), for example by electroplating. The copper surface may then need to be roughened, for example through etching, to allow attachment of the adhesion layer.

In an alternative embodiment the solid substrate comprises ceramic materials, such as are outlined in U.S.S.N.s 09/235,081; 09/337,086; 09/464,490; 09/492,013; 09/466,325; 09/460,281; 09/460,283; 09/387,691; 09/438,600; 09/506,178; and 09/458,534; all of which are expressly incorporated by reference in their entirety. In this embodiment, the devices are made from layers of green-sheet that have been laminated and sintered together to form a substantially monolithic structure. Green-sheet is a composite material that includes inorganic particles of glass, glass-ceramic, ceramic, or mixtures thereof, dispersed in a polymer binder, and may also include additives such as plasticizers and dispersants. The green-sheet is preferably in the form of sheets that are 50 to 250 microns thick. The ceramic particles are typically metal oxides, such as aluminum oxide or zirconium oxide. An example of such a green-sheet that includes glass-ceramic particles is "AX951" that is sold by E.I. Du Pont de Nemours and Company. An example of a green-sheet that includes aluminum oxide particles is "Ferro Alumina" that is sold by Ferro Corp. The composition of the green-sheet may also be custom formulated to meet particular applications. The green-sheet layers are laminated together and then fired to form a substantially monolithic multilayered structure. The manufacturing, processing, and applications of ceramic green-sheets are described generally in Richard E. Mistler, "Tape Casting: The Basic Process for Meeting the Needs of the Electronics Industry " Ceramic Bulletin, 69(6):1022-1026 (1990), and in U.S. Patent No. 3,991,029, which are incorporated herein by reference.

The method for fabricating devices begins with providing sheets of green-sheet that are preferably 50 to 250 microns thick. The sheets of green-sheet are cut to the desired size, typically 6 inches by 6 inches for conventional processing, although smaller or larger devices may be used as needed. Each green-sheet layer may then be textured using various techniques to form desired structures, such as vias, channels, or cavities, in the finished multilayered structure. In preferred embodiments an array of cavities or wells are constructed. In this preferred embodiment, each well has a micro-electrode in electrical

contact with a linking moiety (preferably polyacrylamide gel), wherein the binding molecule is bound to the linking moiety.

Various techniques may be used to texture a green-sheet layer. For example, portions of a green-sheet layer may be punched out to form vias or channels. This operation may be accomplished using conventional multilayer ceramic punches, such as the Pacific Trinetics Corp. Model APS-8718 Automated Punch System. Instead of punching out part of the material, features, such as channels and wells may be embossed into the surface of the green-sheet by pressing the green-sheet against an embossing plate that has a negative image of the desired structure. Texturing may also be accomplished by laser tooling with a laser via system, such as the Pacific Trinetics LVS-3012.

Next, a wide variety of materials may be applied, preferably in the form of thick-film pastes, to each textured green-sheet layer. For example, electrically conductive pathways may be provided by depositing metal-containing thick-film pastes onto the green-sheet layers. Thick-film pastes typically include the desired material, which may be either a metal or a dielectric, in the form of a powder dispersed in an organic vehicle, and the pastes are designed to have the viscosity appropriate for the desired deposition technique, such as screen-printing. The organic vehicle may include resins, solvents, surfactants, and flow-control agents. The thick-film paste may also include a small amount of a flux, such as a glass frit, to facilitate sintering. Thick-film technology is further described in J.D. Provance, "Performance Review of Thick Film Materials," *Insulation/Circuits* (April, 1977) and in Morton L. Topfer, *Thick Film Microelectronics, Fabrication, Design, and Applications* (1977), pp. 41-59, which are incorporated herein by reference.

The porosity of the resulting thick-film can be adjusted by adjusting the amount of organic vehicle present in the thick-film paste. Specifically, the porosity of the thick-film can be increased by increasing the percentage of organic vehicle in the thick-film paste. Similarly, the porosity of a green-sheet layer can be increased by increasing the proportion of organic binder. Another way of increasing porosity in thick-films and green-sheet layers is to disperse within the organic vehicle, or the organic binder, another organic phase that is not soluble in the organic vehicle. Polymer microspheres can be used advantageously for this purpose.

To add electrically conductive pathways, the thick film pastes typically include metal particles, such as silver, platinum, palladium, gold, copper, tungsten, nickel, tin, or alloys thereof. Silver pastes are preferred. Examples of suitable silver pastes are silver conductor composition numbers 7025 and 7713 sold by E.I. Du Pont de Nemours and Company.

The thick-film pastes are preferably applied to a green-sheet layer by screen-printing. In the screen-printing process, the thick-film paste is forced through a patterned silk screen so as to be deposited onto the green-sheet layer in a corresponding pattern. Typically, the silk screen pattern is created photographically by exposure to a mask. In this way, conductive traces may be applied to a surface of a green sheet layer. Vias present in the green-sheet layer may also be filled with thick-film pastes. If filled with thick-filled pastes containing electrically conductive materials, the vias can serve to provide electrical connections between layers and/or into wells within an array of wells.

After the desired structures are formed in each layer of green-sheet, preferably a layer of adhesive is applied to either surface of the green-sheet. Preferably, the adhesive is a room-temperature adhesive. Such room-temperature adhesives have glass transition temperatures below room temperature, *i.e.*, below about 20° C, so that they can bind substrates together at room temperature. Moreover, rather than undergoing a chemical change or chemically reacting with or dissolving components of the substrates, such room-temperature adhesives bind substrates together by penetrating into the surfaces of the substrates. Sometimes such room-temperature adhesives are referred to as "pressure-sensitive adhesives." Suitable room-temperature adhesives are typically supplied as water-based emulsions and are available from Rohm and Haas, Inc. and from Air Products, Inc. For example, a material sold by Air Products, Inc. as "Flexcryl 1653" has been found to work well.

The room-temperature adhesive may be applied to the green-sheet by conventional coating techniques. To facilitate coating, it is often desirable to dilute the supplied pressure-sensitive adhesive in water, depending on the coating technique used, and on the viscosity and solids loading of the starting material. After coating, the room-temperature adhesive is allowed to dry. The dried thickness of the film of room-temperature adhesive is preferably in the range of 1 to 10 microns, and the thickness should be uniform over the entire surface of the green-sheet. Film thicknesses that exceed 15 microns are undesirable. With such thick films of adhesive voiding or delamination can occur during firing due to the large quantity of

organic material that must be removed. Films that are less than about 0.5 microns thick when dried are too thin, because they provide insufficient adhesion between the layers of green-sheet.

From among conventional coating techniques, spin-coating and spraying are the preferred methods. If spin-coating is used, it is preferable to add 1 gram of deionized water for every 10 grams of "Flexcryl 1653." If spraying is used, a higher dilution level is preferred to facilitate ease of spraying. Additionally, when room-temperature adhesive is sprayed on, it is preferable to hold the green-sheet at an elevated temperature, e.g., about 60 to 70° C, so that the material dries nearly instantaneously as it is deposited onto the green-sheet. The instantaneous drying results in a more uniform and homogeneous film of adhesive.

After the room-temperature adhesive has been applied to the green-sheet layers, the layers are stacked together to form a multilayered green-sheet structure. Preferably, the layers are stacked in an alignment die, so as to maintain the desired registration between the structures of each layer. When an alignment die is used, alignment holes must be added to each green-sheet layer.

Typically, the stacking process alone is sufficient to bind the green-sheet layers together when a room-temperature adhesive is used. In other words, little or no pressure is required to bind the layers together. However, in order to effect a more secure binding of the layers, the layers are preferably laminated together after they are stacked.

The lamination process involves the application of pressure to the stacked layers. For example, in the conventional lamination process, a uniaxial pressure of about 1000 to 1500 psi is applied to the stacked green-sheet layers that is then followed by an application of an isostatic pressure of about 3000 to 5000 psi for about 10 to 15 minutes at an elevated temperature, such as 70° C. Adhesives do not need to be applied to bind the green-sheet layers together when the conventional lamination process is used.

However, pressures less than 2500 psi are preferable in order to achieve good control over the dimensions of such structures as internal or external cavities and channels. Even lower pressures are more desirable to allow the formation of larger structures, such as cavities and channels. For example, if a lamination pressure of 2500 psi is used, the size of well-formed internal cavities and channels is typically limited to no larger than roughly 20 microns. Accordingly, pressures less than 1000 psi are more preferred, as such pressures

generally enable structures having sizes greater than about 100 microns to be formed with some measure of dimensional control. Pressures of less than 300 psi are even more preferred, as such pressures typically allow structures with sizes greater than 250 microns to be formed with some degree of dimensional control. Pressures less than 100 psi, which are referred to herein as "near-zero pressures," are most preferred, because at such pressures, few limits exist on the size of internal and external cavities and channels that can be formed in the multilayered structure.

The pressure is preferably applied in the lamination process by means of a uniaxial press.

Alternatively, pressures less than about 100 psi may be applied by hand.

As with semiconductor device fabrication, many devices may be present on each sheet.

Accordingly, after lamination the multilayered structure may be diced using conventional green-sheet dicing or sawing apparatus to separate the individual devices. The high level of peel and shear resistance provided by the room-temperature adhesive results in the occurrence of very little edge delamination during the dicing process. If some layers become separated around the edges after dicing, the layers may be easily re-laminated by applying pressure to the affected edges, for example by hand, without adversely affecting the rest of the device.

The final processing step is firing to convert the laminated multilayered green-sheet structure from its "green" state to form the finished, substantially monolithic, multilayered structure. The firing process occurs in two important stages as the temperature is raised. The first important stage is the binder burnout stage that occurs in the temperature range of about 250 to 500° C, during which the other organic materials, such as the binder in the green-sheet layers and the organic components in any applied thick-film pastes, are removed from the structure.

The sintering stage, the next important stage of firing, occurs at a higher temperature, in which the inorganic particles sinter together. Sintering results in the multilayered becoming densified and substantially monolithic. The sintering temperature used depends on the nature of the inorganic particles present in the green-sheet. For many types of ceramics, appropriate sintering temperatures range from about 950 to about 1600° C, depending on the

material. For example, for green-sheet containing aluminum oxide, sintering temperatures between 1400 and 1600° C are typical. Other ceramic materials, such as silicon nitride, aluminum nitride, and silicon carbide require higher sintering temperatures, namely 1700 to 2200° C. For green-sheet with glass-ceramic particles, a sintering temperature in the range of 750 to 950° C is typical. Glass particles generally require sintering temperatures in the range of only about 350 to 700° C. Finally, metal particles may require sintering temperatures anywhere from 550 to 1700° C, depending on the metal.

Typically, the devices are fired for a period of about 4 hours to about 12 hours or more, depending on the material used. Generally, the firing should be of a sufficient duration so as to remove the organic materials from the structure, and to completely sinter the inorganic particles. In particular, polymers are present as a binder in the green-sheet and in the room-temperature adhesive, and the firing should be of sufficient temperature and duration to decompose these polymers, and to allow for their removal from the multilayered structure.

Typically, the multilayered structure undergoes a reduction in volume during the firing process. During the binder burnout phase, a small volume reduction of about 0.5 to 1.5% is normally observed. At higher temperatures, during the sintering stage, a further volume reduction of about 14 to 17% is typically observed.

The volume change due to firing, on the other hand, can be controlled. In particular, to match volume changes in two materials, such as green-sheet and thick-film paste, one should match: (1) the particle sizes; and (2) the percentage of organic components, such as binders, which are removed during the firing process. Volume changes need not be matched exactly, but any mismatch will typically result in internal stresses in the device. Additionally, symmetrical processing, placing the identical material or structure on opposite sides of the device can, to some extent, compensate for shrinkage stresses caused by mismatched materials. Too great a mismatch in either sintering temperatures or volume changes may result in defects in or failure of some or all of the device. For example, the device may delaminate into its individual layers, or it may become warped or distorted.

Dissimilar materials, such as thick-film pastes or other green-sheet layers, may be added prior to or after the firing process. As noted above, preferably any dissimilar materials added to the green-sheet layers prior to firing, and the dissimilar materials and the green-sheet

layers are co-fired. The benefit of co-firing is that the added materials are sintered to the green-sheet layers and become integral to the substantially monolithic microfluidic device. However, to be co-fireable, the added materials should have sintering temperatures and volume changes due to firing that are matched with those of the green-sheet layers. Sintering temperatures are largely material-dependent, so that matching sintering temperatures simply requires proper selection of materials. For example, although silver is the preferred metal for providing electrically conductive pathways, if the green-sheet layers contain alumina particles, which require a sintering temperature in the range of 1400 to 1600° C, some other metal, such as platinum, must be used due to the relatively low melting point of silver (961° C).

Alternatively, the addition of other substrates or joining of two post-sintered (*i.e.* post fired) pieces can be done using any variety of adhesive techniques, including those outlined herein. For example, two "halves" of a device can be glued or fused together. Thus, in a preferred embodiment polyacrylamide gel and biological components, which are not stable at high temperature, can be sandwiched in between the two halves. Alternatively, ceramic devices comprising open channels or wells can be made, additional substrates or materials placed into the devices, and then they may be sealed with other materials.

The substrates of the invention can form microfluidic cassettes or devices that can be used to effect a number of manipulations on a sample to ultimately result in cell detection or quantification. These manipulations can include cell handling (cell concentration, cell lysis, cell removal, cell separation, etc.). separation of the desired cell from other sample components, chemical or enzymatic reactions on the cell, detection of the cellsj or other components, etc. The devices of the invention can include one or more wells for sample manipulation, waste or reagents; microchannels to and between these wells, including microchannels containing electrophoretic separation matrices; valves to control fluid movement; on-chip pumps such as electroosmotic, electrohydrodynamic, or electrokinetic pumps; and detection systems. The devices of the invention can be configured to manipulate one or multiple samples or analytes.

The devices of the invention can be made in a variety of ways, as will be appreciated by those in the art. See for example WO96/39260, directed to the formation of fluid-tight electrical conduits; U.S. Patent No. 5,747,169, directed to sealing; EP 0637996 B1; EP

0637998 B1; WO96/39260; WO97/16835; WO98/13683; WO97/16561; WO97/43629; WO96/39252; WO96/15576; WO96/15450; WO97/37755; and WO97/27324; and U.S. Patent Nos. 5,304,487; 5,071531; 5,061,336; 5,747,169; 5,296,375; 5,110,745; 5,587,128; 5,498,392; 5,643,738; 5,750,015; 5,726,026; 5,35,358; 5,126,022; 5,770,029; 5,631,337; 5,569,364; 5,135,627; 5,632,876; 5,593,838; 5,585,069; 5,637,469; 5,486,335; 5,755,942; 5,681,484; and 5,603,351, all of which are hereby incorporated by reference. Suitable fabrication techniques again will depend on the choice of substrate, but preferred methods include, but are not limited to, a variety of micromachining and microfabrication techniques, including film deposition processes such as spin coating, chemical vapor deposition, laser fabrication, photolithographic and other etching techniques using either wet chemical processes or plasma processes, embossing, injection molding and bonding techniques (see U.S. Patent No. 5,747,169, hereby incorporated by reference). In addition, there are printing techniques for the creation of desired fluid guiding pathways; that is, patterns of printed material can permit directional fluid transport. Thus, the build-up of "ink" can serve to define a flow channel. In addition, the use of different "inks" or "pastes" can allow different portions of the pathways having different flow properties. For example, materials can be used to change solute/solvent RF values (the ratio of the distance moved by a particular solute to that moved by a solvent front). For example, printed fluid guiding pathways can be manufactured with a printed layer or layers comprised of two different materials, providing different rates of fluid transport. Multi-material fluid guiding pathways can be used when it is desirable to modify retention times of reagents in fluid guiding pathways. Furthermore, printed fluid guiding pathways can also provide regions containing reagent substances, by including the reagents in the "inks" or by a subsequent printing step. See for example U.S. Patent No. 5,795,453, herein incorporated by reference in its entirety.

In addition, it should be understood that while most of the discussion herein is directed to the use of planar substrates with microchannels and wells, other geometries can be used as well. For example, two or more planar substrates can be stacked to produce a three dimensional device, that can contain microchannels flowing within one plane or between planes; similarly, wells may span two or more substrates to allow for larger sample volumes. Thus for example, both sides of a substrate can be etched to contain microchannels; see for

example U.S. Patent Nos. 5,603,351 and 5,681,484, both of which are hereby incorporated by reference.

Thus, the devices of the invention may include at least one microchannel or flow channel that allows the flow of sample from the sample inlet port to the other components or modules of the system. The collection of microchannels and wells is sometimes referred to in the art as a "mesoscale flow system". As will be appreciated by those in the art, the flow channels may be configured in a wide variety of ways, depending on the use of the channel. For example, a single flow channel starting at the sample inlet port may be separated into a variety of smaller channels, such that the original sample is divided into discrete subsamples for parallel processing or analysis. Alternatively, several flow channels from different modules, for example the sample inlet port and a reagent storage module may feed together into a mixing chamber or a reaction chamber. As will be appreciated by those in the art, there are a large number of possible configurations; what is important is that the flow channels allow the movement of sample and reagents from one part of the device to another. For example, the path lengths of the flow channels may be altered as needed; for example, when mixing and timed reactions are required, longer and sometimes tortuous flow channels can be used.

In general, the microfluidic devices of the invention are generally referred to as "mesoscale" devices. The devices herein are typically designed on a scale suitable to analyze microvolumes, although in some embodiments large samples (e.g. cc's of sample) may be reduced in the device to a small volume for subsequent analysis. That is, "mesoscale" as used herein refers to chambers and microchannels that have cross-sectional dimensions on the order of 0.1 μm to 500 μm. The mesoscale flow channels and wells have preferred depths on the order of 0.1 μm to 100 μm, typically 2-50 μm. The channels have preferred widths on the order of 2.0 to 500 μm, more preferably 3-100 μm. For many applications, channels of 5-50 μm are useful. However, for many applications, larger dimensions on the scale of millimeters may be used. Similarly, chambers (sometimes also referred to herein as "wells") in the substrates often will have larger dimensions, on the scale of a few millimeters. In addition to the flow channel system, the devices of the invention are configured to include one or more of a variety of components, herein referred to as "modules", that will be present on any given device depending on its use. These modules include, but are not limited to:

sample inlet ports; sample introduction or collection modules; cell handling modules (for example, for cell lysis, cell removal, cell concentration, cell separation or capture, cell growth, etc.); separation modules, for example, for electrophoresis, dielectrophoresis, gel filtration, ion exchange/affinity chromatography (capture and release) etc.; reaction modules for chemical or biological alteration of the sample, including amplification of the nucleic acids, chemical, physical or enzymatic cleavage or alteration of the sample components, or chemical modification of the target; fluid pumps; fluid valves; thermal modules for heating and cooling; storage modules for assay reagents; mixing chambers; and detection modules.

In a preferred embodiment, the devices of the invention include at least one sample inlet port for the introduction of the sample to the device. This may be part of or separate from a sample introduction or collection module; that is, the sample may be directly fed in from the sample inlet port to a separation chamber, or it may be pretreated in a sample collection well or chamber.

In a preferred embodiment, the devices of the invention include a sample collection module, which can be used to concentrate or enrich the sample if required; for example, see U.S. Patent No. 5,770,029, including the discussion of enrichment channels and enrichment means.

In a preferred embodiment, the devices of the invention include a cell handling module. Thus, for example, the detection of particular antibodies in blood can require the removal of the blood cells for efficient analysis, or the cells (and/or nucleus) may be lysed. In this context, "cells" include eukaryotic and prokaryotic cells, and viral particles that may require treatment prior to analysis, such as the release of nucleic acid from a viral particle prior to detection of target sequences. In addition, cell handling modules may also utilize a downstream means for determining the presence or absence of cells. Suitable cell handling modules include, but are not limited to, cell lysis modules, cell removal modules, cell concentration modules, and cell separation or capture modules. In addition, as for all the modules of the invention, the cell handling module is in fluid communication via a flow channel with at least one other module of the invention.

In a preferred embodiment, the cell handling module includes a cell lysis module. As is known in the art, cells may be lysed in a variety of ways, depending on the cell type. In one embodiment, as described in EP 0 637 998 B1 and U.S. Patent No. 5,635,358, hereby

incorporated by reference, the cell lysis module may comprise cell membrane piercing protrusions that extend from a surface of the cell handling module. As fluid is forced through the device, the cells are ruptured. Similarly, this may be accomplished using sharp edged particles trapped within the cell handling region. Alternatively, the cell lysis module can comprise a region of restricted cross-sectional dimension, which results in cell lysis upon pressure.

In a preferred embodiment, the cell lysis module comprises a cell lysing agent, such as guanidium chloride, chaotropic salts, enzymes such as lysozymes, etc. In some embodiments, for example for blood cells, a simple dilution with water or buffer can result in hypotonic lysis. The lysis agent may be solution form, stored within the cell lysis module or in a storage module and pumped into the lysis module. Alternatively, the lysis agent may be in solid form, that is taken up in solution upon introduction of the sample.

The cell lysis module may also include, either internally or externally, a filtering module for the removal of cellular debris as needed. This filter may be microfabricated between the cell lysis module and the subsequent module to enable the removal of the lysed cell membrane and other cellular debris components; examples of suitable filters are shown in EP 0 637 998 B1, incorporated by reference.

In a preferred embodiment, the cell handling module includes a cell separation or capture module. This embodiment utilizes a cell capture region comprising binding sites capable of reversibly binding a cell surface molecule to enable the selective isolation (or removal) of a particular type of cell from the sample. These binding moieties may be immobilized either on the surface of the module or on a particle trapped within the module (i.e. a bead) by physical absorption or by covalent attachment. Suitable binding moieties will depend on the cell type to be isolated or removed, and generally includes antibodies and other binding ligands, such as ligands for cell surface receptors, etc. as outlined herein Thus, a particular cell type may be removed from a sample prior to further handling, or the assay is designed to specifically bind the desired cell type, wash away the non-desirable cell types, followed by either release of the bound cells by the addition of reagents or solvents, physical removal (i.e. higher flow rates or pressures), or even in situ lysis.

Alternatively, a cellular "sieve" can be used to separate cells on the basis of size. This can be done in a variety of ways, including protrusions from the surface that allow size exclusion, a series of narrowing channels, a weir, or a diafiltration type setup.

In a preferred embodiment, the cell handling module includes a cell removal module. This may be used when the sample contains cells that are not required in the assay or are undesirable. Generally, cell removal will be done on the basis of size exclusion as for "sieving", above, with channels exiting the cell handling module that are too small for the cells.

In a preferred embodiment, the cell handling module includes a cell concentration module. As will be appreciated by those in the art, this is done using "sieving" methods, for example to concentrate the cells from a large volume of sample fluid prior to lysis.

In some embodiments of the present invention, the microelectrodes project from the surface of the substrate, with such projections extending between 5×10^{-8} and 1×10^{-5} cm from the surface of the supporting substrate. In other embodiments, the microelectrodes comprise a flat disk of conductive material that is embedded in the supporting substrate and is exposed at the substrate surface, with the supporting substrate acting as an insulator in the spaces between the microelectrodes.

In a preferred embodiment of the present invention the microelectrodes comprise a gold or platinum conductor and a glass or silicon insulator. In alternative embodiments, the microelectrodes comprise conductor substances such as solid or porous foils or films of silver, titanium, or copper, or metal oxides, metal nitrides, metal carbides, carbon, graphite, or combinations thereof. In additional embodiments, the microelectrodes comprise substrate and/or insulator substances such as plastic, rubber, fabric, ceramics, or combinations thereof. The microelectrodes of the present invention preferably have an exposed conductive surface of from about $0.01 \mu m^2$ to $0.5 cm^2$. In a preferred embodiment, the exposed conductive material has an area of from about 100 to $160{,}000 \mu m^2$.

One embodiment of the present invention is shown in Figure 1A, wherein the microelectrode comprises a glass capillary tube 1, containing an ultra fine platinum wire 2, to which a transition wire 3 has been soldered 6. The transition wire 3 is soldered 6 in turn to a hookup wire 4, which protrudes from an epoxy plug 5 that seals the capillary tube. Polyacrylamide gel material 7 is packed into a recess etched into the exposed surface of the

platinum wire 2. The polymeric hydrogel pad is preferably at least about 0.1 to 30 μm thick, more preferably at least about 0.5 to 10μm thick, and most preferably about 0.5μm thick.

The apparatus of the present invention comprises at least one counter-electrode. In a preferred embodiment of the present invention, the counter-electrode comprises a conductive material, with an exposed surface that is significantly larger than that of the individual microelectrodes. In a preferred embodiment, the counter electrode comprises platinum wire. In altemative embodiments, the counter electrode comprises solid or porous films of gold, silver, platinum, titanium, or copper, or metal oxides, metal nitrides, metal carbides, carbon, graphite, or combinations thereof.

In other embodiments of the present invention, the apparatus comprises at least one reference electrode. The reference electrode is particularly useful in assays for detecting the change in electrical potential after probe and target molecules are allowed to interact and then determining the number or concentration of bacterial cells in a sample using quantitative methods (e.g., voltammetry). In preferred embodiments, the reference electrode comprises a silver/silver chloride electrode. In altemative embodiments, the reference electrode comprises solid or porous films of gold, platinum, titanium, or copper, or metal oxides, metal nitrides, metal carbides, carbon, graphite, or combinations thereof.

In still further embodiments of the present invention, the apparatus further comprises a plurality of wells each of which encompasses at least one microelectrode in contact with a linker moiety and at least one counter-electrode. The term "wells" is used herein in its conventional sense, to describe a portion of the supporting substrate in which the microelectrode and at least one counter-electrode are contained in a defined volume; said wells can protrude from the surface of the supporting substrate, or be embedded therein.

In preferred embodiments of the apparatus of the present invention, the apparatus further comprises a means for killing bacteria bound to specific binding molecules immobilized on linker moieties in contact with microelectrodes following the detection of electrical signal changes at each microelectrode. Means for killing bacterial cells bound to the specific binding molecules include, but are not limited to, heat treatment and irradiation. The killing step permits verification of the signal obtained from the binding of viable bacteria to, for example, antibodies immobilized on a porous hydrogel microelectrode. In preferred embodiments, bacteria bound to specific binding molecules are killed by incubation at a

temperature greater than about 42°C, more preferably greater than about 56°C and most preferably greater than about 65°C. Electrochemical contact between each of the microelectrodes and the counter electrode and/or the reference electrode is advantageously provided using an electrolyte solution in contact with each of the microelectrodes comprising the apparatus of the invention. Electrolyte solutions useful in the apparatus and methods of the invention include any electrolyte solution at physiologically-relevant ionic strength (equivalent to about 0.15 M NaCl) and neutral pH. Examples of electrolyte solutions useful with the apparatus and methods of the invention include, but are not limited to, phosphate buffered saline, HEPES buffered solutions, and sodium bicarbonate buffered solutions. Said electrolyte solutions are in contact with each of the microelectrodes of the apparatus of the invention, the counter-electrode and the reference electrode if provided, thereby providing electrochemical contact between the electrodes.

In preferred embodiments of the present invention, bacterial cells in the sample mixture are detected by detecting an electrical signal using AC impedance. In other embodiments, bacterial cells in the sample mixture are detected by detecting an electrical signal using an electrical detection method selected from the group consisting of impedance spectroscopy, cyclic voltammetry, AC voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, other steady-state or transient measurement methods, and combinations thereof.

In one embodiment of the apparatus of the present invention, the means for producing electrical impedance at each microelectrode is accomplished using a Model 1260 Impedance/Gain-Phase Analyser with Model 1287 Electrochemical Interface (Solartron Inc., Houston, TX). Other electrical impedance measurement means include, but are not limited to, transient methods using AC signal perturbation superimposed upon a DC potential applied to an electrochemical cell such as AC bridge and AC voltammetry. The measurements can be conducted at a certain particular frequency that specifically produces electrical signal changes that are readily detected or otherwise determined to be advantageous. Such particular frequencies are advantageously determined by scanning frequencies to ascertain the frequency producing, for example, the largest difference in electrical signal. The means for detecting changes in impedance at each microelectrode as a result of antibody-bacterial cell

binding can be accomplished by using any of the above-described instruments and analytical methods.

The apparatus and methods of the present invention are useful for the electrical detection or differentiation of bacteria using binding molecules specific for a particular bacterium and electrical detection techniques. In one method of the invention, AC impedance is measured at a plurality of microelectrodes in contact with linker moieties to which a polyclonal antibody having specificity to a plurality of antigens of a particular bacterium has been immobilized. The microelectrodes are then exposed to a sample mixture containing bacterial cells, and changes in AC impedance resulting from antibody-bacterial cell binding are then detected at each of the microelectrodes.

In other embodiments, the apparatus and methods are used for electrical differentiation of bacterial cell types using at least two subsets of linker moieties to which antibodies having distinct specificities to at least two different bacterial cell types (*e.g.*, species, subspecies, or strain) have been immobilized. In one method of the invention, AC impedance is measured at a plurality of microelectrodes wherein at least two subsets of linker moieties to which antibodies having distinct specificities to at least two different bacterial cell types have been immobilized. The microelectrodes are then exposed to a sample mixture containing at least two bacterial cell types, and changes in AC impedance resulting from antibody-bacterial cell binding to each subset of microelectrodes are detected.

In some embodiments of the method of the present invention, bacterial detection is accomplished or enhanced by the addition of an antibody molecule labeled with an electrochemical reporter, having specificity for a desired target bacterial cell. Binding molecules that are labeled with an electrochemical reporter (also referred to herein as electron transfer moieties) useful in the methods of the present invention may be prepared by labeling suitable binding molecules with any electrochemically-distinctive redox reporter that does not interfere with the molecular interaction to be detected. In preferred embodiments of the method of the present invention, a second antibody or other specific binding molecule, which does not interfere with the interaction between the bacteria and the specific binding molecule immobilized on the linker moiety (e.g, a primary antibody), is labeled with electrochemical reporter groups comprising a transition metal complex or organic redox compounds, most preferably containing a ransition metal ion that is ruthenium, cobalt, iron, or osmium.

"Electron transfer moiety" or "ETM" herein is meant a compound which is capable of reversibly, semi-reversibly, or irreversibly transferring one or more electrons. The terms "electron donor moiety", "electron acceptor moiety", and "electron transfer moieties" or grammatical equivalents herein refers to molecules capable of electron transfer under certain conditions. It is to be understood that electron donor and acceptor capabilities are relative; that is, a molecule which can lose an electron under certain experimental conditions will be able to accept an electron under different experimental conditions. It is to be understood that the number of possible electron donor moieties and electron acceptor moieties is very large, and that one skilled in the art of electron transfer compounds will be able to utilize a number of compounds in the present invention. Preferred electron transfer moieties include, but are not limited to, transition metal complexes, organic electron transfer moieties, and electrodes.

In a preferred embodiment, the electron transfer moieties are transition metal complexes. Transition metals include those whose atoms have a partial or complete d shell of electrons; elements having the atomic numbers 21-30, 39-48, 57-80 and the lanthanide series. Suitable transition metals for use in the invention include, but are not limited to, cadmium (Cd), copper (Cu), cobalt (Co), palladium (Pd), zinc (Zn), iron (Fe), ruthenium (Ru), rhodium (Rh), osmium (Os), rhenium (Re), platinium (Pt), scandium (Sc), titanium (Ti), Vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), Molybdenum (Mo), technetium (Tc), tungsten (W), and iridium (Ir). That is, the first series of transition metals, the platinum metals (Ru, Rh, Pd, Os, Ir and Pt), along with Fe, Re, W, Mo and Tc, are preferred. Particularly preferred are ruthenium, rhenium, osmium, platinium, cobalt and iron.

The transition metals are complexed with a variety of ligands, generally depicted herein as "L", to form suitable transition metal complexes, as is well known in the art. Suitable ligands fall into two categories: ligands which use nitrogen, oxygen, sulfur, carbon or phosphorus atoms (depending on the metal ion) as the coordination atoms (generally referred to in the literature as sigma (σ) donors) and organometallic ligands such as metallocene ligands (generally referred to in the literature as pi (π) donors, and depicted herein as L_m). Suitable nitrogen donating ligands are well known in the art and include, but are not limited to, NH_2; NHR; NRR'; pyridine; pyrazine; isonicotinamide; imidazole; bipyridine and substituted derivatives of bipyridine; terpyridine and substituted derivatives; phenanthrolines, particularly 1,10-phenanthroline (abbreviated phen) and substituted

derivatives of phenanthrolines such as 4,7-dimethylphenanthroline and dipyridol[3,2-a:2',3'-c]phenazine (abbreviated dppz); dipyridophenazine; 1,4,5,8,9,12-hexaazatriphenylene (abbreviated hat); 9,10-phenanthrenequinone diimine (abbreviated phi); 1,4,5,8-tetraazaphenanthrene (abbreviated tap); 1,4,8,11-tetra-azacyclotetradecane (abbreviated cyclam) and isocyanide. Substituted derivatives, including fused derivatives, may also be used. In some embodiments, porphyrins and substituted derivatives of the porphyrin family may be used. See for example, Comprehensive Coordination Chemistry, Ed. Wilkinson et al., Pergammon Press, 1987, Chapters 13.2 (pp73-98), 21.1 (pp. 813-898) and 21.3 (pp 915-957), all of which are hereby expressly incorporated by reference.

Suitable sigma donating ligands using carbon, oxygen, sulfur and phosphorus are known in the art. For example, suitable sigma carbon donors are found in Cotton and Wilkenson, Advanced Organic Chemistry, 5th Edition, John Wiley & Sons, 1988, hereby incorporated by reference; see page 38, for example. Similarly, suitable oxygen ligands include crown ethers, water and others known in the art. Phosphines and substituted phosphines are also suitable; see page 38 of Cotton and Wilkenson.

The oxygen, sulfur, phosphorus and nitrogen-donating ligands are attached in such a manner as to allow the heteroatoms to serve as coordination atoms.

In a preferred embodiment, organometallic ligands are used. In addition to purely organic compounds for use as redox moieties, and various transition metal coordination complexes with δ-bonded organic ligand with donor atoms as heterocyclic or exocyclic substituents, there is available a wide variety of transition metal organometallic compounds with π-bonded organic ligands (see Advanced Inorganic Chemistry, 5th Ed., Cotton & Wilkinson, John Wiley & Sons, 1988, chapter 26; Organometallics, A Concise Introduction, Elschenbroich et al., 2nd Ed., 1992, VCH; and Comprehensive Organometallic Chemistry II, A Review of the Literature 1982-1994, Abel et al. Ed., Vol. 7, chapters 7, 8, 10 & 11, Pergamon Press, hereby expressly incorporated by reference). Such organometallic ligands include cyclic aromatic compounds such as the cyclopentadienide ion [$C_5H_5(-1)$] and various ring substituted and ring fused derivatives, such as the indenylide (-1) ion, that yield a class of bis(cyclopentadieyl)metal compounds, (i.e. the metallocenes); see for example Robins et al., J. Am. Chem. Soc. 104:1882-1893 (1982); and Gassman et al., J. Am. Chem. Soc. 108:4228-4229 (1986), incorporated by reference. Of these, ferrocene [$(C_5H_5)_2Fe$] and its

derivatives are prototypical examples which have been used in a wide variety of chemical (Connelly et al., Chem. Rev. 96:877-910 (1996), incorporated by reference) and electrochemical (Geiger et al., Advances in Organometallic Chemistry 23:1-93; and Geiger et al., Advances in Organometallic Chemistry 24:87, incorporated by reference) electron transfer or "redox" reactions. Metallocene derivatives of a variety of the first, second and third row transition metals are potential candidates as redox moieties. Other potentially suitable organometallic ligands include cyclic arenes such as benzene, to yield bis(arene)metal compounds and their ring substituted and ring fused derivatives, of which bis(benzene)chromium is a prototypical example, Other acyclic π-bonded ligands such as the allyl(-1) ion, or butadiene yield potentially suitable organometallic compounds, and all such ligands, in conjuction with other π-bonded and δ-bonded ligands constitute the general class of organometallic compounds in which there is a metal to carbon bond. Electrochemical studies of various dimers and oligomers of such compounds with bridging organic ligands, and additional non-bridging ligands, as well as with and without metal-metal bonds are potential candidate redox moieties.

As described herein, any combination of ligands may be used. Preferred combinations include: a) all ligands are nitrogen donating ligands; b) all ligands are organometallic ligands; and c) the ligand at the terminus of the conductive oligomer is a metallocene ligand and the ligand provided by the binding ligand is a nitrogen donating ligand, with the other ligands, if needed, are either nitrogen donating ligands or metallocene ligands, or a mixture.

In addition to transition metal complexes, other organic electron donors and acceptors may be used in the invention. These organic molecules include, but are not limited to, riboflavin, xanthene dyes, azine dyes, acridine orange, *N,N'*-dimethyl-2,7-diazapyrenium dichloride (DAP^{2+}), methylviologen, ethidium bromide, quinones such as N,N'-dimethylanthra(2,1,9-*def*:6,5,10-*d'e'f'*)diisoquinoline dichloride ($ADIQ^{2+}$); porphyrins ([meso-tetrakis(N-methyl-x-pyridinium)porphyrin tetrachloride], varlamine blue B hydrochloride, Bindschedler's green; 2,6-dichloroindophenol, 2,6-dibromophenolindophenol; Brilliant crest blue (3-amino-9-dimethyl-amino-10-methylphenoxyazine chloride), methylene blue; Nile blue A (aminoaphthodiethylaminophenoxazine sulfate), indigo-5,5',7,7'-tetrasulfonic acid, indigo-5,5',7-trisulfonic acid; phenosafranine, indigo-5-monosulfonic acid;

safranine T; bis(dimethylglyoximato)-iron(II) chloride; induline scarlet, neutral red, anthracene, coronene, pyrene, 9-phenylanthracene, rubrene, binaphthyl, DPA, phenothiazene, fluoranthene, phenanthrene, chrysene, 1,8-diphenyl-1,3,5,7-octatetracene, naphthalene, acenaphthalene, perylene, TMPD and analogs and subsitituted derivatives of these compounds.

In one embodiment, the electron donors and acceptors are redox proteins as are known in the art. However, redox proteins in many embodiments are not preferred.

In other embodiments of the present invention, molecules are labeled with the following non-limiting examples of electrochemically-active moieties: 1,4-benzoquinone, ferrocene, tetracyanoquinodimethane, N,N,N',N'-tetramethyl p-phenylenediamine, tetrathiafulvalene, 9-aminoacridine, acridine orange, aclarubicin, daunomycin, doxorubicin, pirarubicin, ethidium bromide, ethidium monoazide, chlortetracycline, tetracycline, minocycline, Hoechst 33258, Hoechst 33342, 7-aminoactinomycin D, Chromomycin A_3, mithramycin A, Vinblastine, Rifampicin, $Os(bipyridine)_2(dipyridophenazine)_2^+$, $Co(bipyridine)_3^{3+}$, or Fe-bleomycin.

The electrochemically-active moiety comprising the electrochemically active reporter-labeled molecule used in certain embodiments of the methods of the present invention is optionally linked to the antibody molecule through a linker, preferably having a length of from about 10 to about 20 Angstroms. The linker can be an organic moiety such as a hydrocarbon chain $(CH_2)_n$, wherein n is an integer from 1 to about 20, or can comprise an ether; ester, carboxyamide, or thioether moiety, or a combination thereof. The linker can also be an inorganic moiety such as siloxane (O-Si-O). The length of the linker is selected so that the electrochemically-active moiety does not interfere with the molecular interaction to be detected.

In other embodiments of the present invention, electrical techniques can be used to detect the binding of a bacterial cell to an antibody (comprising a "capture" antibody) immobilized on the surface of the microelectrode, and the binding of a secondary, electrochemically labeled antibody (comprising a "detection" antibody) to the bacterial cells bound by the capture antibody. Such electrical techniques include, but are not limited to, impedance spectroscopy, cyclic voltammetry, AC voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance,

potential step method, potentiometric measurements, amperometric measurements, current step method, other steady-state or transient measurement methods and combinations thereof.

The preferred embodiments of the present invention are best understood by referring to Figures 1-6 and Examples 1-3. The Examples, which follow, are illustrative of specific embodiments of the invention, and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention.

EXAMPLE 1

Preparation of Streptavidin-Modified Porous Hydrozel Microelectrodes

Streptavidin-modified porous hydrogel microelectrodes used for detecting viable bacteria were prepared as follows. Ultra-fine platinum wire having a diameter of 50μm was inserted into glass capillary tubing having a diameter of 2 mm and sealed by heating to form a solidmicroelectrode structure. The tip of the structure was then polished with gamma alumina powder (CH Instruments, Inc., Austin, TX) to expose a flat disk of the platinum wire. Microelectrodes were initially polished with 0.3 μm gamma alumina powder, rinsed with deionized water, and then polished with 0.005μm powder. Following polishing, the microelectrodes were ultrasonically cleaned for 2 min. in deionized water, soaked in 1N HNO_3 for 20 min., vigorously washed in deionized water, immersed in acetone for 10 min., and again washed vigorously in deionized water. Through the use of micromanufacturing techniques employed in the fabrication of semiconductors, modifications of this procedure can be applied to the preparation of microelectrodes of a size required for the construction of alternative embodiments of the microelectrodes, such as bioarray chips (see co-owned and co-pending U.S. Patent App. Serial Nos. 09/458,501 and 09/458,533, incorporated by reference).

Porous hydrogel microelectrodes were prepared from the above-described microelectrodes as follows. The exposed flat disk of platinum of each microelectrode was etched in hot aqua regia to form a recess (i.e., micropore dent) of a specified depth. The depth of the recess was controlled by the length of time that the platinum disk was exposed to the etching material, with the depth of the micropore dent ranging from several microns to more than 1mm. The recess thus formed was then packed with 1μL of streptavidin-modified polyacrylamide gel material (Figure 1B) and polymerized under UV irradiation in a

Stratalinker (Stratagene, La Jolla, CA) for 20 min. to form a porous hydrogel microelectrode (Figure 2). A porous hydrogel microelectrode having a diameter of about 260μm was used as described below in Example 2. Prior to attachment of antibodies, porous hydrogel microelectrodes were ultrasonically treated in acetone and then in 1M HNO_3 for 10 min.

Streptavidin-modified polyacrylamide gel material was prepared as follows. A streptavidin solution was prepared by mixing 10 μ L of an N-acyloxysuccinimide solution (prepared by dissolving 10 mg of N-acyloxysuccinimide in 72μL of DMSO) with 200μL of streptavidin stock solution (prepared by dissolving 10 mg streptavidin in 2.5 mL phosphate buffered saline (PBS) at pH 7.6). This mixture was incubated at room temperature for 2-3 hours and then centrifuged for 2 minutes at 13,000 rpm in a conventional desktop eppendorf microcentrifuge to remove precipitated material. An acrylamide solution was prepared by first dissolving 25 mg bis-acrylamide in 6 mL PBSt pH 7.6, dissolving 475 mg acrylamide monomer in this solution, and then filtering the mixture through a 5 micron filter. To prepare streptavidin-modified polyacrylamide gel material, 290 ~L of the acrylamide solution was mixed with 210μL of the streptavidin solution, and 150μL of this solution was added to 0.6μL of 1mM methylene blue and 1.8μL TEMED, poured into the recesses in the microelectrodes and allowed to polymerize prior to use.

EXAMPLE 2

Immobilization of Antibodies on Streptavidin-Modified Porous Hydrogel Microelectrodes

To attach antibodies to the microelectrodes prepared in Example 1, the microelectrodes were incubated at room temperature or at about 25°C for 1.5 hours in 500μL of a solution consisting of 50μg/mL of biotinylated polyclonal anti *E. coli* antibody (Virostat, Portland, ME) having specificity for a plurality of *E. coli* antigens. Following attachment of the antibodies, the microelectrodes were vigorously washed in PBS prior to use.

EXAMPLE 3

Electrical Detection of Viable Bacteria

The presence of viable bacteria in a sample solution was detected using the porous hydrogel microelectrodes prepared as described above by measuring changes in AC

impedance. AC impedance was measured using a Model 1260 Impedance/GainPhase Analyser with Model 1287 Electrochemical Interface (Solartron Inc.). A platinum wire having a surface area larger than the hydrogel porous microelectrode was used as the counter electrode. Impedance measurements were made under open circuit voltage (OCV) conditions in PBS and samples were excited at an amplitude of 20 mV.

Following vigorous washing with PBS, the baseline AC impedance for the microelectrodes prepared in Example 2 was measured in PBS as electrolyte solution. The microelectrode was then incubated at room temperature or at 25°C in a sample solution containing 10^8 colony-forming units (cfu)/mL *E. coli* for 15 hours. Following this incubation, AC impedance was once again measured. Figures 4A-4B show the relationship between the measured capacitance and the AC frequency (Figure 4A) and measured resistance and AC frequency (Figure 4B) for the porous hydrogel microelectrode before (curve 1) and after (curve 2) incubation with the *E. coli* sample. These plots indicate that, following incubation in the sample solution, there was a decrease in capacitance and a significant increase in resistance. These results suggest that the bacteria in the sample solution were bound to microelectrode-immobilized antibody, blocking electron transfer which was detected using the porous hydrogel microelectrode.

To examine whether electrical detection could be used to differentiate between viable and heat-killed bacteria, viable bacteria bound on a porous hydrogel microelectrode were killed by incubation at 65°C for 5 min. Following this incubation, AC impedance was once again measured. Figures 5A-5B show the relationship between the measured capacitance and the AC frequency (Figure 5A) and measured resistance and AC frequency (Figure 5B) for the porous hydrogel microelectrode after incubation with the *E. coli* sample (curve 1) and following heat killing (curve 2). These plots indicate that, following the high temperature treatment, there is a significant change in impedance. The AC impedance following heat killing returned to levels near those observed prior to incubating the microelectrodes with the bacterial sample. The resulting AC impedance did not, however, return to the pre-incubation baseline value. This suggested that electrical detection using porous hydrogel microelectrodes could be applied to the differentiation between viable and heat-killed bacteria.

To ensure that the change in AC impedance observed after bacterial heat killing was not due to detachment of the cells from the porous hydrogel microelectrodes, the microelectrodes were incubated at 4°C temperature for 18 hours in a solution consisting of O.5mL of 5Oμg/mL of a horse radish peroxidase (HRP)-labeled polyclonal anti-*E. coli* antibody (Virostat). Following incubation, AC impedance was once again measured. Figures 6A-6B show a graph of capacitance versus frequency (Figure 6A) and resistance versus frequency (Figure 6B) for the microelectrode after heat killing (curve 2) and after incubation with the HRP-labeled polyclonal anti-*E. coli* antibody (curve 3). The alteration in AC impedance observed following incubation with the HRP-labeled anti-*E. coli* antibody demonstrated that *E. coli* cells did not detach from the porous hydrogel microelectrode following thermal killing.

Figures 6A-6B show plots of capacitance versus frequency (Figure 6A) and resistance versus frequency (Figure 6B) over a frequency range of 1000 Hz to 0.1 MHz for the microelectrode after incubation with *E. coli* (curve 1) and after incubation with the HRP-labeled polyclonal anti-*E. coli*- antibody (curve 2). This data corresponds to that represented in Figures 5A and 5B (curves 1 and 3), and demonstrated that the AC impedance measured for microelectrodes with immobilized, viable *E. coli* differs from that measured for microelectrodes having bound thereto heat-killed *E. coli* that were reacted with an HRP-labeled anti-*E. coli* antibody.

Figure 7 illustrates the specificity of the electrical impedance measurements for specific binding of bacteria to antibodies that recognize bacterial cell antigens. Microelectrodes prepared using the anti-*E. coli* polyclonal antibodies described above were immobilized on a streptavidin-acrylamide deposited microelectrode. After vigorously washing the electrodes with PBS, the baseline AC impedance for microelectrodes with immobilized anti-*E. coli* polyclonal antibodies was measured in PBS (curve 2). The electrode was then immersed for 15 hours in a sample solution containing 10^8 cfu/mL *Bacillus subtilis*. Following exposure to the sample solution, AC impedance was again measured (curve 3). No change in the impedance in the presence (curve 3) or absence (curve 2) of bacterial cells was observed. These results demonstrate that the microelectrodes of the invention are capable of specific detection of microorganisms without detectable cross-reactivity between unrelated species.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

What is claimed is:

1. An apparatus for the electrical detection of bacterial cells in a sample mixture, comprising:

(a) a supporting substrate,

(b) one or a plurality of microelectrodes in contact with the supporting substrate,

(c) one or a plurality of linking moieties in contact with the microelectrodes and to which specific binding molecules have been immobilized,

(d) at least one counter-electrode in electrochemical contact with the microelectrodes,

(e) a means for producing an electrical signal at each microelectrode,

(f) a means for detecting changes in the electrical signal at each microelectrode, and

(g) an electrolyte solution in contact with the microelectrodes, the linking moieties, and the counter-electrode,

wherein bacterial cells in the sample mixture are detected by detecting a difference in the electrical signal at each microelectrode in the presence and absence of the sample mixture in contact with the microelectrodes.

2. The apparatus of Claim 1, wherein the linking moieties comprise polyacrylamide gel, agarose gel, polyethylene glycol, cellulose gel, sol gel, or combinations thereof.

3. The apparatus of Claim 2, wherein the linking moieties comprise polyacrylamide gel.

4. The apparatus of Claim 1, wherein the linking moieties comprise a conjugated polymer or copolymer film.

5. The apparatus of Claim 4, wherein the conjugated polymer or copolymer film is polypyrrole, polythiphene, polyaniline, polyfuran, polypyridine, polycarbazole, polyphenylene, poly(phenylenvinylene), polyfluorene, polyindole, or a derivative, copolymer, or combinations thereof.

6. The apparatus of Claim 1, wherein the linking moieties comprise a neutral pyrrole matrix.

7. The apparatus of Claim 1, wherein the supporting substrate comprises ceramic, glass, silicon, fabric, rubber, plastic, or combinations thereof.

8. The apparatus of Claims 1, wherein the microelectrodes comprise a conductive material and an insulating material.

9. The apparatus of Claim 8, wherein the conductive material is solid or porous gold, silver, platinum, titanium, copper, or metal oxide, metal nitride, metal carbide, carbon, graphite, or combinations thereof.

10. The apparatus of Claim 9, wherein the conductive material is platinum.

11. The apparatus of Claim 9, wherein the conductive material is gold.

12. The apparatus of Claim 8, wherein the insulating material is glass, silicon, plastic, rubber, fabric, ceramic, or combinations thereof.

13. The apparatus of Claim 12, wherein the insulating material is silicon.

14. The apparatus of Claim 12, wherein the insulating material is glass.

15. The apparatus of Claim 8, wherein the conductive material is embedded in the supporting substrate and the supporting substrate comprises the insulating material.

16. The apparatus of Claim 1, further comprising at least one reference electrode.

17. The apparatus of Claim 16, wherein the reference electrode comprises a conductive material and an insulating material.

18. The apparatus of Claim 17, wherein the conductive material is solid or porous gold, silver, platinum, titanium, copper, or metal oxide, metal nitride, metal carbide, carbon, graphite, or combinations thereof.

19. The apparatus of Claim 17, wherein the conductive material is silver/silver chloride.

20. The apparatus of Claim 17, wherein the insulating material is glass, silicon, plastic, rubber, fabric, ceramic, or combinations thereof.

21. The apparatus of Claim 1, wherein the supporting substrate further comprises a plurality of wells, each of which encompasses at least one microelectrode in contact with a linker moiety and at least one counter-electrode.

22. The apparatus of Claim 1, wherein the specific binding molecules are proteins or peptides.

23. The apparatus of Claim 1, wherein the specific binding molecules are antibodies.

24. The apparatus of Claim 23, wherein the antibodies are a polyclonal antisera, polyclonal antibodies, or F(ab), F(ab)', $F(ab)_2$, or F_v fragments thereof.

25. The apparatus of Claim 23, wherein the antibodies are monoclonal antibodies, or F(ab), F(ab)', $F(ab)_2$, or F_v fragments thereof.

26. The apparatus of Claim 23, wherein the antibodies are F(ab) fragments or single-chain F_v fragments produced by *in vitro* libraries.

27. The apparatus of Claim 1, wherein the specific binding molecules are nucleic acids, oligonucleotides, or combinations thereof.

28. The apparatus of Claim 27, wherein the specific binding molecules are aptamers.

29. The apparatus of Claim 1, wherein the specific binding molecules comprise a natural products library, a phage display library, or a combinatorial library.

30. The apparatus of Claim 1, wherein the linking moieties further comprise streptavidin and the specific binding molecules are biotinylated.

31. The apparatus of Claim 1, further comprising a means for killing the bacteria in the sample mixture.

32. The apparatus of Claim 31, wherein the bacteria in the sample mixture are killed by incubating the apparatus at a temperature greater than about 42°C.

33. The apparatus of Claim 32, wherein the bacteria in the sample mixture are killed by exposing the apparatus to UV irradiation.

34. The apparatus of Claim 1, wherein a second specific binding molecule is contacted with bacterial cells in the sample mixture and said second specific binding molecule is labeled.

35. The apparatus of Claim 1, wherein bacterial cells in the sample mixture are detected by using an electrical detection method selected from the group consisting of impedance spectroscopy, cyclic voltammetry, AC voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, other steady-state or transient measurement methods, and combinations thereof.

36. A method for the electrical detection of bacterial cells in a sample mixture, comprising:

(a) detecting an electrical signal in one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized,

(b) exposing the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized to a sample mixture containing bacterial cells,

(c) detecting an electrical signal in the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized,

(d) comparing the electrical signal detected in step (c) with that detected in step (a), and

(e) determining whether the electrical signal detected in step (c) is different from the electrical signal detected in step (a).

37. A method for the electrical detection of viable bacterial cells in a sample mixture, comprising:

(a) exposing one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized with a sample mixture containing bacterial cells,

(b) detecting an electrical signal in the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized,

(c) killing the bacteria in the sample mixture exposed to the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized,

(d) detecting an electrical-signal in the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized,

(e) comparing the electrical signal detected in step (d) with that detected in step (b), and

(f) determining whether the electrical signal detected in step (d) is different from the electrical signal detected in step (b).

38. The method of Claim 37, wherein the sample mixture contains viable bacterial cells, nonviable bacterial cells, or combinations thereof.

39. The method of Claim 37, wherein the bacteria in the sample mixture are killed by incubating the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules are immobilized at a temperature greater than about 42°C.

40. The apparatus of Claim 39, wherein the bacteria in the sample mixture are killed by exposing the one or a plurality of linker moieties in contact with microelectrodes and to which specific binding molecules are immobilized to UV irradiation.

41. A method for the electrical detection of bacterial cells of a particular bacterial species, subspecies, or strain in a sample mixture, comprising:

(a) detecting an electrical signal in one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized, wherein the specific binding molecules have a specificity for the bacterial cells of a particular bacterial species, subspecies, or strain to be detected,

(b) exposing the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized with a sample mixture containing bacterial cells of a particular species, subspecies, or strain,

(c) detecting an electrical signal in the one or a plurality of microelectrodes in contact with linker moieties to which specific binding molecules have been immobilized,

(d) comparing the electrical signal detected in step (c) with that detected in step (a), and

(e) determining whether the electrical signal detected in step (c) is different from the electrical signal detected in step (a).

42. The method of Claim 41, wherein the sample mixture contains a plurality of bacterial species, subspecies, or strains.

43. The method of Claims 36, 37, or 41, wherein the bacterial cells in the sample mixture are detected by detecting an electrical signal using an electrical detection method selected from the group consisting of impedance spectroscopy, cyclic voltammetry, AC voltammetry, pulse voltammetry, square wave voltammetry, AC voltammetry, hydrodynamic modulation

voltammetry, conductance, potential step method, potentiometric measurements, amperometric measurements, current step method, other steady-state or transient measurement methods, or combinations thereof.

44. The method of Claims 36, 37, or 41, wherein the electrical detection method is AC impedance that is measured over a range of AC frequencies.

45. The method of Claims 36, 37, or 41, wherein the electrical detection method is AC impedance that is measured by transient methods with AC signal perturbation superimposed upon a DC potential applied to an electrochemical cell.

46. The method of Claims 36, 37, or 41, wherein the electrical detection method is AC impedance that is measured by impedance analyzer, lock-in amplifier, AC bridge, AC voltammetry, or combinations thereof.

FIG._1A

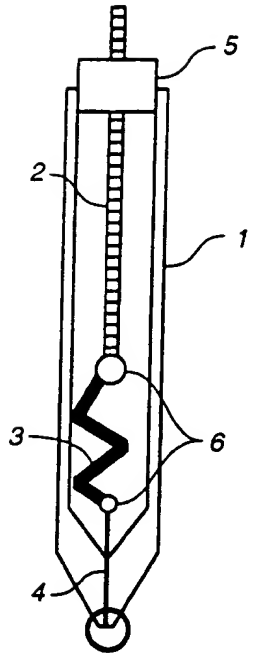

FIG._1B

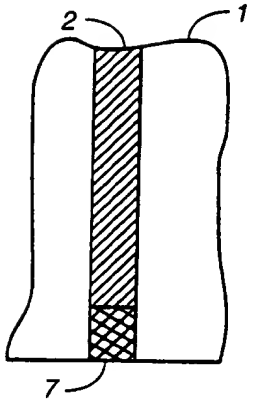

FIG._2

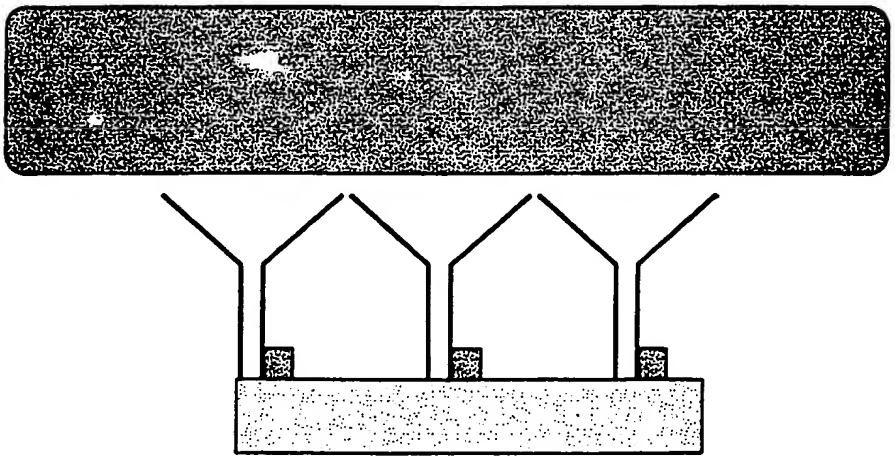

FIG._3

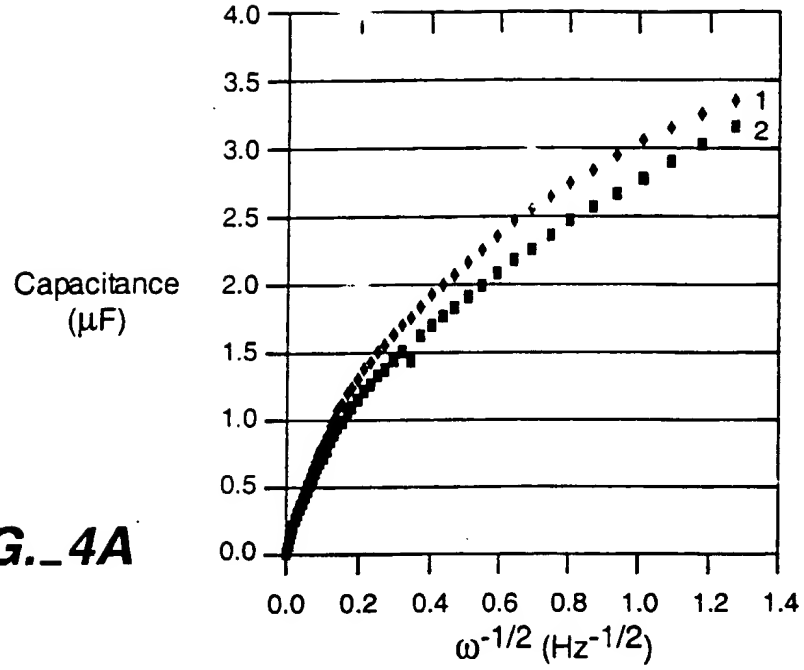
Capacitance
(μF)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1
2
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
ω-1/2 (Hz-1/2)

FIG._4A

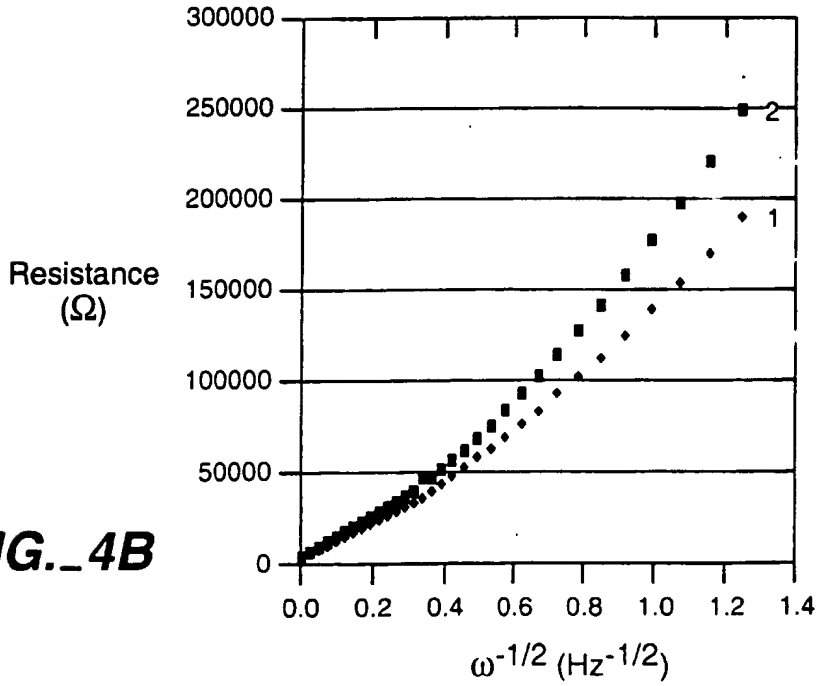
Resistance
(Ω)
300000
250000
200000
150000
100000
50000
0
2
1
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
ω-1/2 (Hz-1/2)

FIG._4B

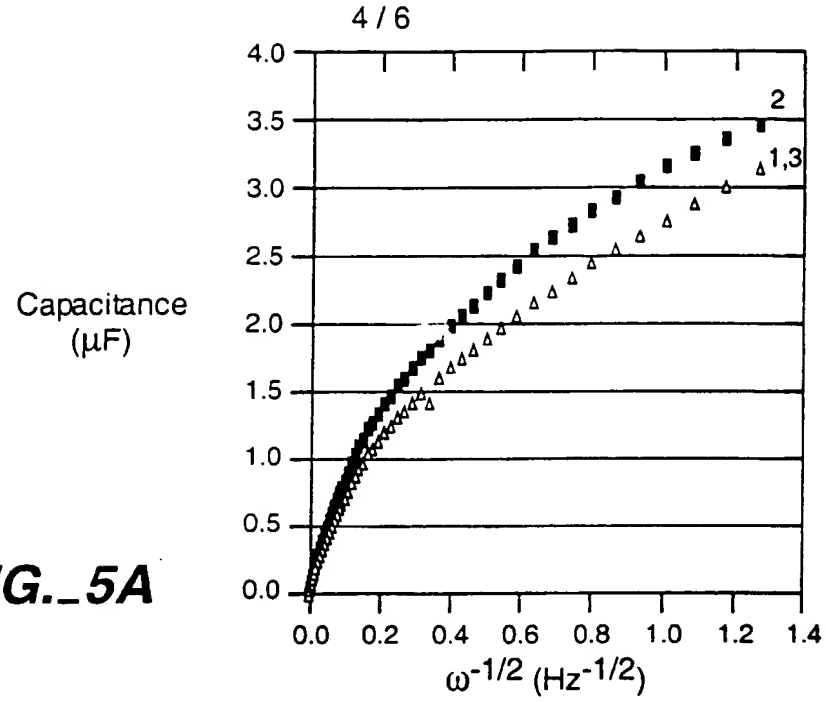
Capacitance
(μF)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2
1,3
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
ω-1/2 (Hz-1/2)

FIG._5A

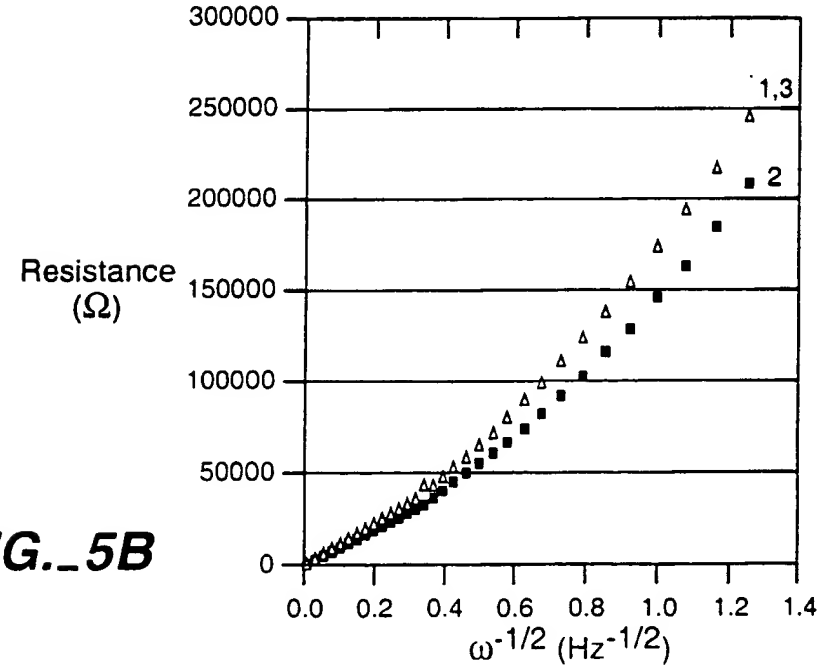
Resistance
(Ω)
300000
250000
200000
150000
100000
50000
0
1,3
2
0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4
ω-1/2 (Hz-1/2)

FIG._5B

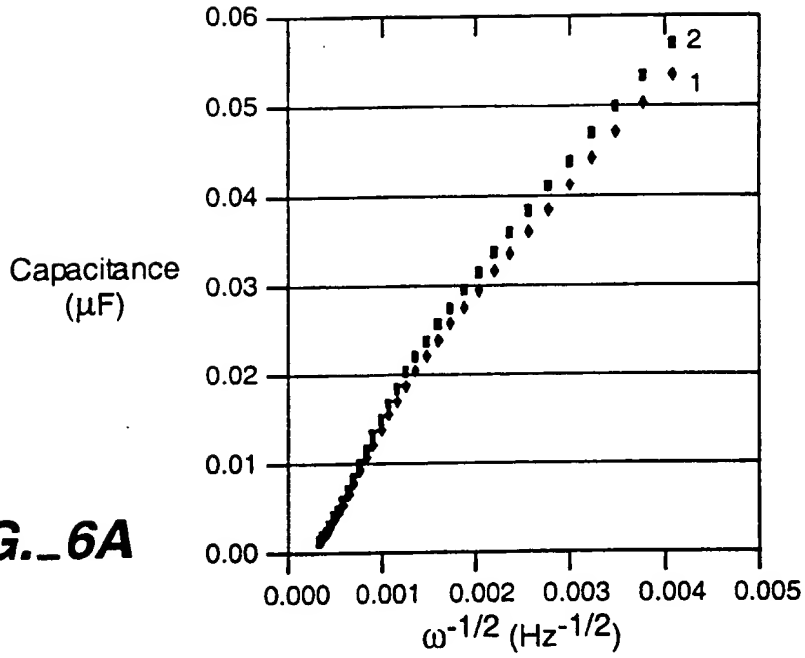
Capacitance
(μF)
0.06
0.05
0.04
0.03
0.02
0.01
0.00
0.000
0.001
0.002
0.003
0.004
0.005
ω-1/2 (Hz-1/2)
1
2

FIG._6A

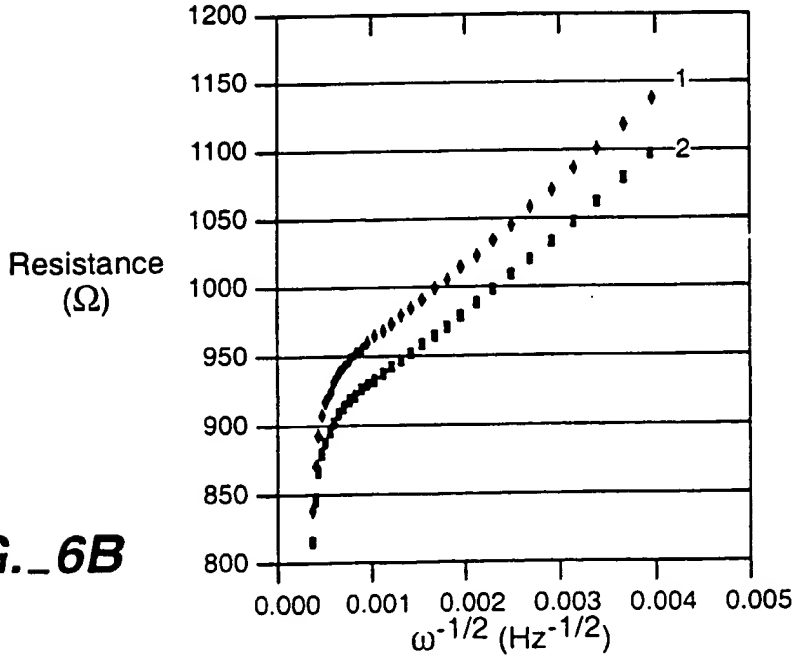
Resistance
(Ω)
1200
1150
1100
1050
1000
950
900
850
800
0.000
0.001
0.002
0.003
0.004
0.005
ω-1/2 (Hz-1/2)
1
2

FIG._6B

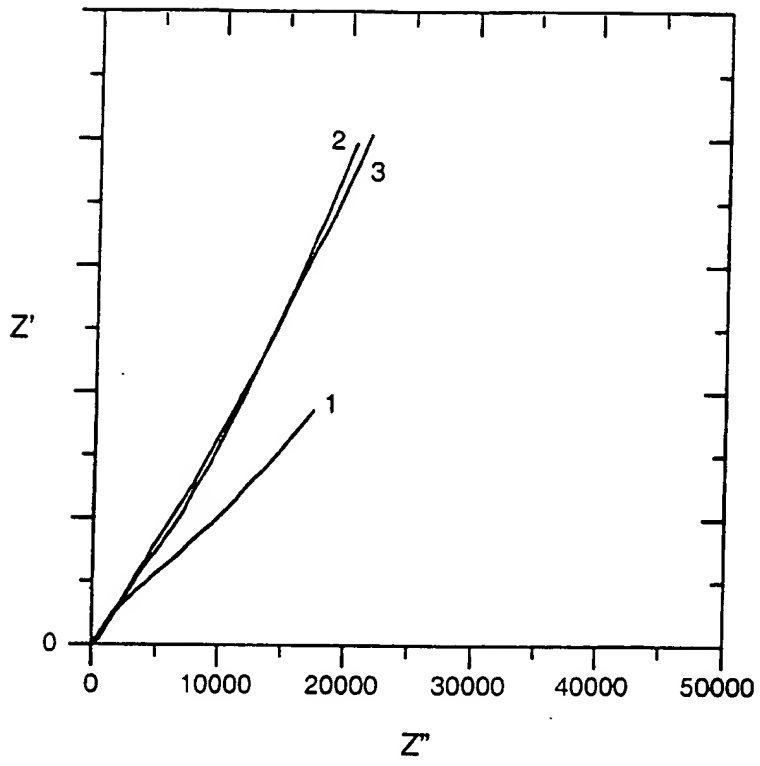

FIG._7